Direct Measurement of the pK_a of Aspartic Acid 26 in *Lactobacillus casei* Dihydrofolate Reductase: Implications for the Catalytic Mechanism

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ABSTRACT: The ionization state of aspartate 26 in *Lactobacillus casei* dihydrofolate reductase has been investigated by selectively labeling the enzyme with [$^{13}C\gamma$] aspartic acid and measuring the ^{13}C chemical shifts in the apo, folate−enzyme, and dihydrofolate−enzyme complexes. Our results indicate that no aspartate residue has a pK_a greater than ∼4.8 in any of the three complexes studied. The resonance of aspartate 26 in the dihydrofolate−enzyme complex has been assigned by site-directed mutagenesis; aspartate 26 is found to have a pK_a value of less than 4 in this complex. Such a low pK_a value makes it most unlikely that the ionization of this residue is responsible for the observed pH profile of hydride ion transfer [apparent pK_a = 6.0; Andrews, J., Fierke, C. A., Birdsall, B., Ostler, G., Feeney, J., Roberts, G. C. K., and Benkovic, S. J. (1989) *Biochemistry 28*, 5743−5750]. Furthermore, the downfield chemical shift of the Asp 26 $^{13}C\gamma$ resonance in the dihydrofolate−enzyme complex provides experimental evidence that the pteridine ring of dihydrofolate is polarized when bound to the enzyme. We propose that this polarization of dihydrofolate acts as the driving force for protonation of the electron-rich O4 atom which occurs in the presence of NADPH. After this protonation of the substrate, a network of hydrogen bonds between O4, N5 and a bound water molecule facilitates transfer of the proton to N5 and transfer of a hydride ion from NADPH to the C6 atom to complete the reduction process.

The enzyme dihydrofolate reductase (DHFR[1]) is responsible for the conversion of dihydrofolate and (in some species) folate to tetrahydrofolate using NADPH as a coenzyme. Although a number of hypotheses have been put forward, the exact manner by which substrate reduction takes place has yet to be conclusively demonstrated. Central to all proposed catalytic mechanisms is a conserved carboxylate group at the active site (an aspartate residue in bacterial and a glutamate residue in mammalian DHFRs). The importance of this residue (Asp 26 in *Lactobacillus casei* DHFR) has been deduced primarily from kinetic and structural data (*1*−*3*). It is located in a hydrophobic pocket where it is hydrogen bonded to the 2-NH_2 and N3 of folate and to a conserved water molecule (Wat 253) as illustrated in Figure 1; it is clearly important in binding and orienting the substrate, but a direct role in catalysis has also been proposed. The rate of hydride ion transfer from NADPH to dihydrofolate (FH_2) is pH-dependent, with an apparent pK_a of 6.0 in *L. casei* DHFR (*4*). Since this aspartate is the only ionizable amino acid residue in the active site, it has been proposed that its ionization, with an unusually high pK_a, accounts for this pH profile (*5*, *6*). It is clear that Asp 26 is not close enough to the N5 of FH_2 for direct proton transfer, and indirect pathways of proton transfer have been suggested (*6*, *7*). Other mechanisms have discounted the possibility that Asp 26 itself acts as the source of the proton, preferring rather to ascribe it a key role in catalyzing the proton transfer (*8*, *9*). To date, attempts to determine the ionization state of Asp 26 have involved indirect measurements (*10*) which have met with varied success. In this paper we describe the use of ^{13}C NMR spectroscopy of selectively [$^{13}C\gamma$-Asp]-labeled DHFR to determine directly the ionization state of Asp 26 in a number

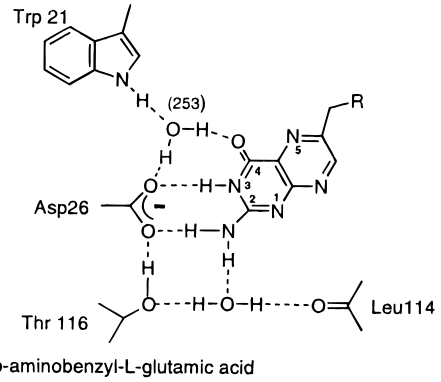

FIGURE 1: Proposed hydrogen bonding interaction for folate bound to *E. coli* DHFR (using *L. casei* numbering in Figure). Diagram adapted from X-ray crystallographic data (*34*, *35*).

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[1] Abbreviations: DHFR, dihydrofolate reductase; FH_2, 7,8-dihydrofolate; FH_4, 5,6,7,8-tetrahydrofolate; H_2NADPH, 1,4,5,6-tetrahydronicotinamide adenine dinucleotide phosphate.

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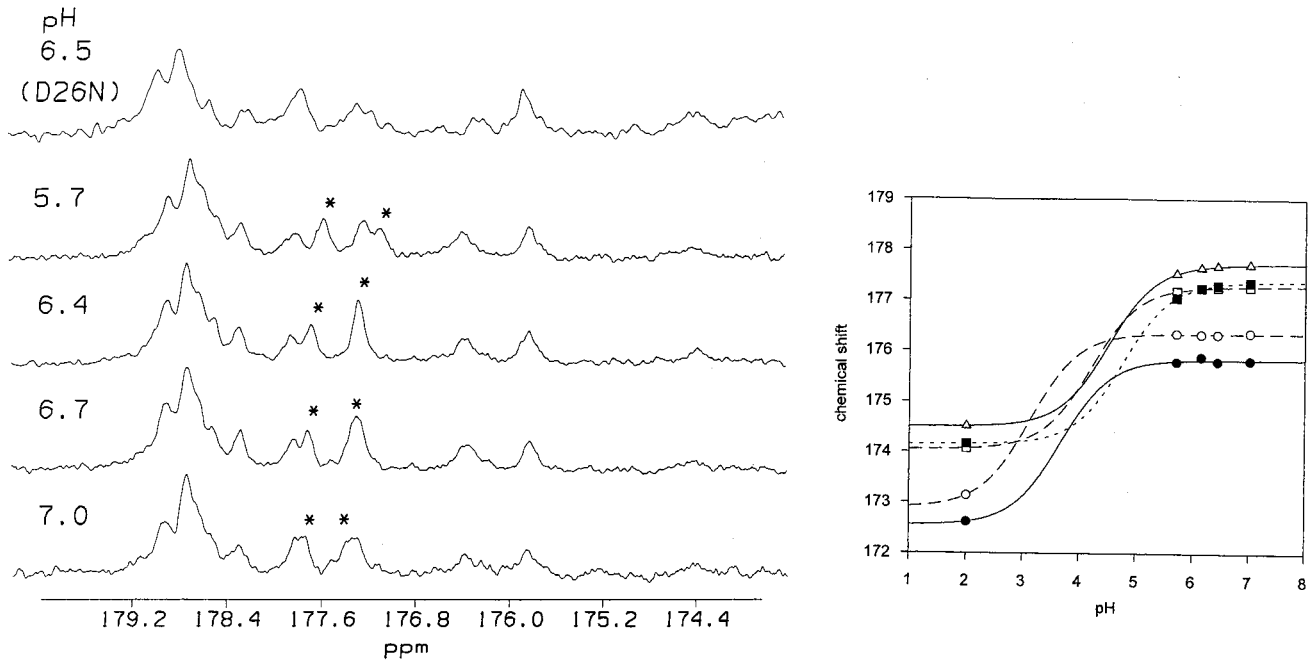

FIGURE 2: Part of the ^{13}C NMR spectrum at 285 K of [$^{13}C\gamma$-Asp]-labeled DHFR apoenzyme at various pH values and on top, the D26N apoenzyme at pH 6.5. The resonances marked with asterisks indicate the Asp resonances with elevated pK_a values. The inset shows the chemical shift versus pH titration profiles for five upfield peaks; △ and ■ denote peaks marked with asterisks.

of enzyme−substrate complexes. On the basis of these and other experimental observations, we are able to put forward a plausible catalytic mechanism.

MATERIALS AND METHODS

Folic acid, dihydrofolate, and dithiothreitol (DTT) were purchased from Sigma Chemical Co. and were of the highest purity commercially available; these ligands were used with no further purification.

NADPH was obtained from Sigma Chemical Co. Tetrahydronicotinamide adenine dinucleotide phosphate (H_2-NADPH) was prepared from NADPH using the method of Biellmann & Jung (*11*) in which NADPH (120 mg) was dissolved in 5 mL of distilled water and hydrogenated using 15 mg of 10% Pd/C catalyst. The reaction was allowed to proceed until the characteristic NADPH absorbance peak at 340 nm was no longer detectable. The mixture was then filtered to remove the solid Pd/C particles. Commercial preparations of NADPH contain a breakdown product that is a potent inhibitor of DHFR activity at low pH (*12*). To obtain pure NADPH and H_2NADPH, we purified samples prior to use by FPLC using a Pharmacia Mono Q anion-exchange column (*13*). H_2NADPH was stored at −70 °C until required. The purity of the H_2NADPH samples was confirmed by measuring the A263/A288 ratio (*11*).

The preparation of [$^{13}C\gamma$-Asp]-labeled DHFR was carried out as previously described (*14*). The DHFR D26N mutant was created by site-directed mutagenesis (*14*, *15*) and the mutant gene subsequently cloned into the pET11a expression vector. The enzymes were purified as described by Dann et al. (*16*). Protein purity was checked by running 12% SDS−PAGE gels, and enzyme concentrations were determined by absorbance spectroscopy (using $\epsilon = 30\,500\ M^{-1}\ cm^{-1}$ at 280 nm (*4*)).

NMR samples consisted of approximately 0.5−1.0 mM labeled enzyme, 200 mM KCl, and 50 mM potassium phosphate in a H_2O solution containing 10% ^2H_2O. For the ligand complexes, two molar equivalents of substrate and coenzyme were added to the enzyme. One-dimensional ^{13}C NMR spectra were recorded at 150.93 MHz on a Bruker AMX-600 spectrometer. For the apoenzyme- and dihydrofolate-containing samples, a temperature of 285 K was used; for other complexes this was increased to 293 K. Typical parameters included 16 K data points, a spectral width of 7576 Hz, an acquisition time of 0.67 s, a relaxation delay of 0.4 s, and 10000−16000 transients for each experiment. Data sets were zero filled to 32 K and multiplied by an exponential function prior to transformation. ^{13}C chemical shift values are reported relative to dioxan.

To stabilize FH_2 during the course of the NMR experiment, we thoroughly deoxygenated the sample by purging it with ultrapure helium followed by argon. Further precautions were taken by the addition of 5 mM DTT and by carrying out all manipulations in a strictly controlled anaerobic environment. The sample was sealed with a rubber septum and microliter additions of concentrated HCl and/or KOH made to adjust the pH, which was measured by monitoring the ^{31}P resonance of the phosphate buffer, previously calibrated as a function of pH. ^1H NMR spectra were recorded after each titration to ensure the presence of free FH_2.

RESULTS

Apoenzyme. The ^{13}C spectra of apo-[$^{13}C\gamma$-Asp]-labeled DHFR at different pH values and the corresponding apo-D26N mutant (pH 6.5) are shown in Figure 2. The spectra show a number of peaks with chemical shifts consistent with that of aspartic acid carboxylate carbons (*17*), spread across a 5 ppm chemical shift range with the greatest overlap occurring at the low-field end of this region. From a cursory inspection of this series spectra it is possible to identify at least 12 resonances from the 15 Asp residues present in *L. casei* DHFR. The resonances of two of these residues

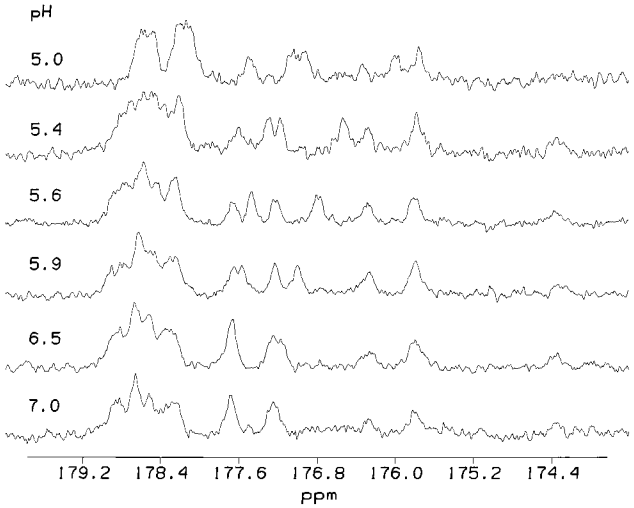

FIGURE 3: Part of the ^{13}C NMR spectrum at 293 K of the 2:1 complex of the folate complex of [$^{13}C\gamma$-Asp]-labeled DHFR at various pH values.

(marked with asterisks) are seen to shift with pH over the range shown, indicating that two aspartic acid residues have slightly elevated pK_a values relative to the others. The instability of apo-DHFR at low pH is such that complete titration curves were not attainable as observed in the inset for Figure 2. However, assuming that the chemical shift difference between a fully protonated and unprotonated aspartic acid γ-carbon is approximately 3.2 ppm (*17*), one can estimate approximate pK_a values of 4.8 and 4.5.

In an attempt to identify the Asp 26 resonance, an identical strategy for selective ^{13}C-labeling with [$^{13}C\gamma$]-aspartic acid was employed for DHFR D26N. Previous NMR and X-ray crystallographic studies of DHFR D26N and the equivalent *Escherichia coli* D27N mutant have shown that the substitution is accompanied by only minor local structural changes. However, as seen in Figure 2, the spectra of the mutant were so similar to those of the wild-type that the resonance of Asp 26 could not be identified with any confidence; it is most likely located in the overlapped group of signals at 178.5−179 ppm.

Folate Complex. Addition of folate to the labeled wild-type enzyme had little effect on the chemical shifts of the resolved resonances (Figure 3), although the quality of the spectra is clearly better than that seen for the apoenzyme, due presumably to the fact that the increased stability of the enzyme−folate complex relative to the apoenzyme enabled us to collect the data at a somewhat higher temperature. As for the apoenzyme, two residues have somewhat higher pK_a values than the remainder; by pH 5, all but three of the residues are beginning to titrate. The fact that neither of the two resonances corresponding to residues with elevated pK_a values show changes in chemical shift on the addition of folate supports the conclusion from the spectra of the apoenzyme of DHFR D26N that neither resonance is that of Asp 26.

Dihydrofolate Complexes. The substrate dihydrofolate is known to degrade over a period of several hours to produce folate, pteridine, and *p*-aminobenzyl glutamate, the process being accelerated at low pH (*18*). We have devised a protocol which dramatically decreases the rate of degradation, so that after 24 h at pH 6, 80% of the FH_2 remains intact. Since FH_2 binds to *L. casei* DHFR at least 30-fold more tightly than any of its breakdown products (*19*, *20*), the presence of excess FH_2 in solution will ensure that the enzyme exists almost exclusively as its FH_2 complex. At the end of each pH titration a ^1H spectrum was recorded to confirm the presence of free FH_2.

The ^{13}C spectra of the binary enzyme−FH_2 complex (Figure 4) had a similar overall appearance to those of the DHFR−folate complexes. However, the spectra of the enzyme−FH_2 complex contain a resonance shifted significantly downfield, to 179.2 ppm, which is seen more clearly in Figure 4A. This resonance is absent in the spectrum of [$^{13}C\gamma$-Asp]-labeled DHFR D26N (Figure 4B), strongly suggesting that it arises from Asp 26. Also, this resonance is not present in the spectra of the enzyme−folate complex and does not titrate in the experimental pH range. From the pH-independent chemical shift of this resonance, we can see that the Asp 26 resonance has barely started to titrate at pH 5 and conclude that the carboxylate of aspartate 26 has a pK_a below 4 in the DHFR−FH_2 complex.

It was not possible to obtain data on the DHFR−FH_2−$NADP^+$ complex as a model of the catalytically functional ternary complex, since the enzyme catalyzes the dismutation of FH_2 to folate and FH_4 (*21*). One alternative coenzyme analogue which has previously been used is tetrahydroNADPH (H_2NADPH). However, in the presence of this analogue, FH_2 was oxidized to folate over a period of time ranging from minutes to hours, making NMR titration experiments impractical. Nonetheless, it was possible to acquire data for one pH value (6.7) prior to the oxidation of FH_2. The spectra of the DHFR−FH_2−H_2NADPH complex and the corresponding complex of the D26N mutant are shown in Figure 5, parts a and b, respectively. A striking feature which sets the wild-type spectrum apart from any of the previous recorded spectra is the presence of an isolated downfield shifted peak at 180.6 ppm. In the corresponding D26N mutant complex this peak is absent. A smaller peak at ∼179.2 ppm which is also present in the wild-type spectrum disappears in the D26N spectrum. This peak is most likely due to the degradation of H_2NADPH which occurs under neutral and acidic conditions (*22*) and which would lead to the formation of the binary FH_2 complex whose Asp 26 resonance is at this position. As the pH for the complex was altered, the downfield peak at 180.6 ppm disappeared, presumably due to the conversion of dihydrofolate to folate (spectra not shown). Thus, the $^{13}C\gamma$ resonance of Asp 26, which is already the lowest-field aspartate carboxyl resonance in the enzyme−FH_2 complex, is shifted still further downfield on addition of a reduced coenzyme analogue.

DISCUSSION

The charge state and pK_a value of the active-site aspartate/glutamate residue of DHFR have been the subject of considerable discussion in the literature, as attempts are made to define the role of this residue in proton transfer during catalysis and in the pH dependence of hydride transfer. Blakley et al. (*10*) employed NMR to probe the ionization state of Glu 30 (human DHFR) indirectly, measuring the chemical shifts of $^{13}C/^{15}N$-labeled folate and FH_2; since no significant chemical shift difference was observed for either of the bound substrates over the pH range of 5−8.5, it was

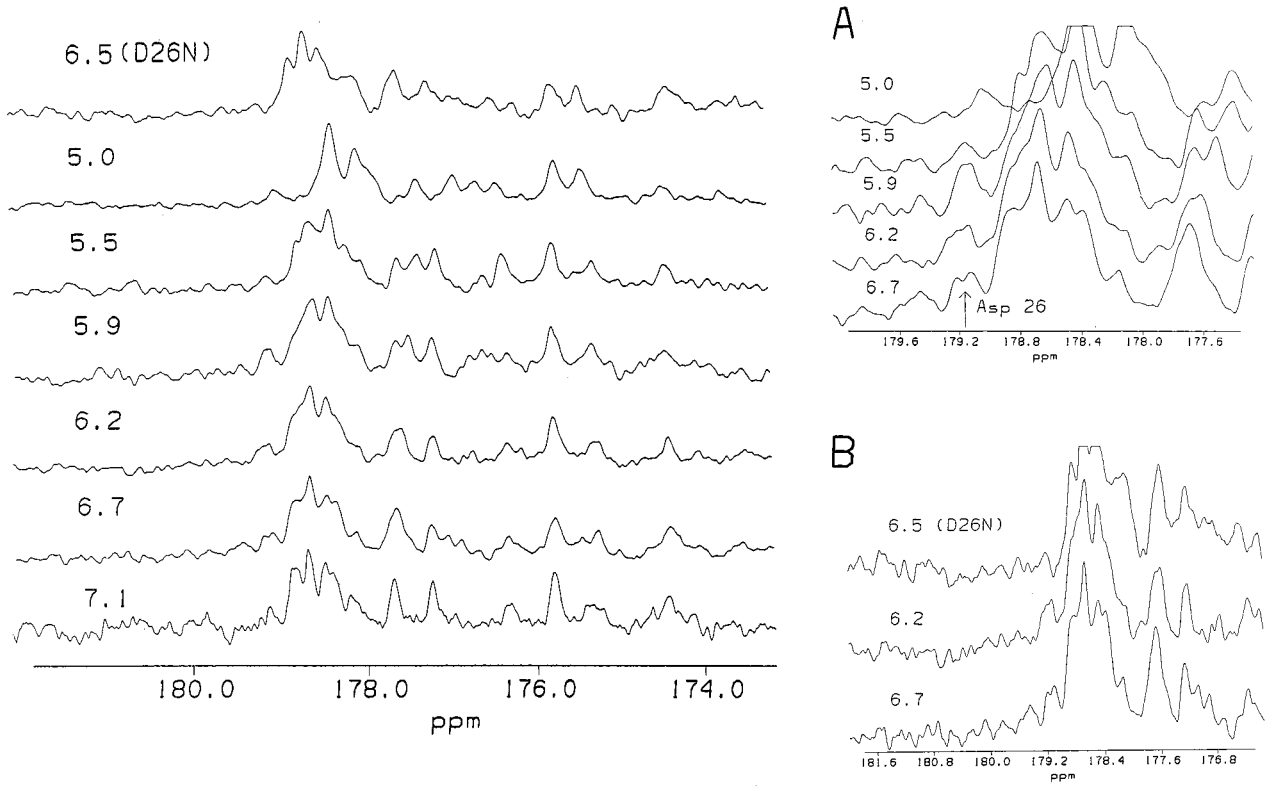

FIGURE 4: Downfield region of the ^{13}C NMR spectrum at 293 K of [$^{13}C\gamma$-Asp]-labeled DHFR in the presence of a 2-fold molar excess of FH_2 at various pH values and D26N DHFR at pH 6.5. Inset A shows the magnification of the Asp 26 peak, and inset B shows the absence of the Asp 26 peak in the D26N spectrum at pH 6.5; for comparison, below is the Asp 26 peak in the wild-type spectrum at pH values 6.2 and 6.7.

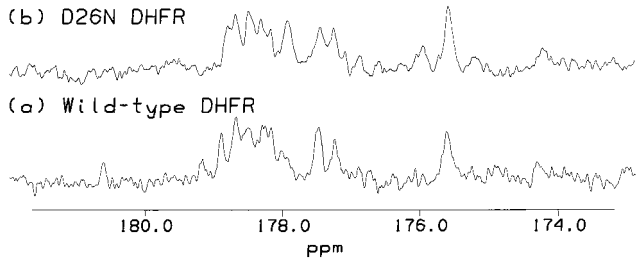

FIGURE 5: Downfield region of the ^{13}C NMR spectrum at 293 K of the complex of [$^{13}C\gamma$-Asp]-labeled DHFR with FH_2 and H_2-NADPH (a) pH 6.7 and (b) D26N pH 6.9.

concluded that the glutamate side chain remained in an ionized state and that folate and FH_2 were bound to human DHFR in the keto tautomeric state. In the work described here, we have been able to obtain direct information on the charge state and pK_a of Asp 26 of *L. casei* DHFR by ^{13}C NMR of selectively labeled enzyme. It is clear from our pH titration studies of the apoenzyme and the DHFR−folate and DHFR−FH_2 complexes that no aspartic acid residue in the enzyme has a pK_a value greater than ~4.8. In the complexes, the resonance of Asp 26 has been specifically assigned by site-directed mutagenesis, and in these cases we can thus conclude with some confidence that this key active-site residue does exist in the ionized (carboxylate) form at pH > 4. Addition of the coenzyme analogue H_2NADPH to the enzyme−FH_2 complex at pH 6.7 to form a model for the catalytic ternary complex leads to a change in chemical shift of the Asp 26 $^{13}C\gamma$ resonance in the direction opposite to that expected for protonation of the carboxyl group. The direct NMR evidence thus strongly suggests that ionization of Asp 26 cannot be responsible for the apparent pK_a value of 6.0 which characterizes the pH dependence of hydride ion transfer for *L. casei* DHFR. This conclusion is in contrast to that recently drawn by Cannon et al. (*23*) from the results of Poisson−Boltzmann electrostatic calculations on the enzyme−FH_2−NADPH complex.

The true origin of this pH dependence is still a matter of debate, but there is increasing support for the idea that it is associated in some manner with the ionization and/or tautomeric state of the substrate. Raman spectroscopy has recently been used to investigate various binary and ternary complexes of *E. coli* DHFR (*24*, *25*). From this work it was concluded that the pK_a of N5 of FH_2 was increased by ~4 units to 6.5 in the ternary DHFR−FH_2−$NADP^+$ complex relative to the binary DHFR−FH_2 and ternary DHFR−FH_2−H_2NADPH complexes. Since this value is the same as that measured from the pH dependence of hydride ion transfer for *E. coli* DHFR (*26*), it was suggested that the protonation state of N5 and not Asp 27 determined the pH dependence of catalysis (*24*). However, the finding that the pK_a of N5 (2.6 in the free substrate) is increased only in the dead-end enzyme−FH_2−$NADP^+$ complex and not in the enzyme−FH_2−H_2NADPH complex, which should be a better model for the catalytically functional ternary complex (at least in terms of the charge state of the nicotinamide ring), is difficult to reconcile with ground-state protonation of N5 as the origin of the pH dependence of catalysis. Cannon et al. (*23*) have calculated the Raman frequencies in a 7,8-dihydropterin-acetate model of the active site and conclude that the observed frequency shift could be due to keto-enol tautomerisation at N3−O4 rather than to N5 protonation.

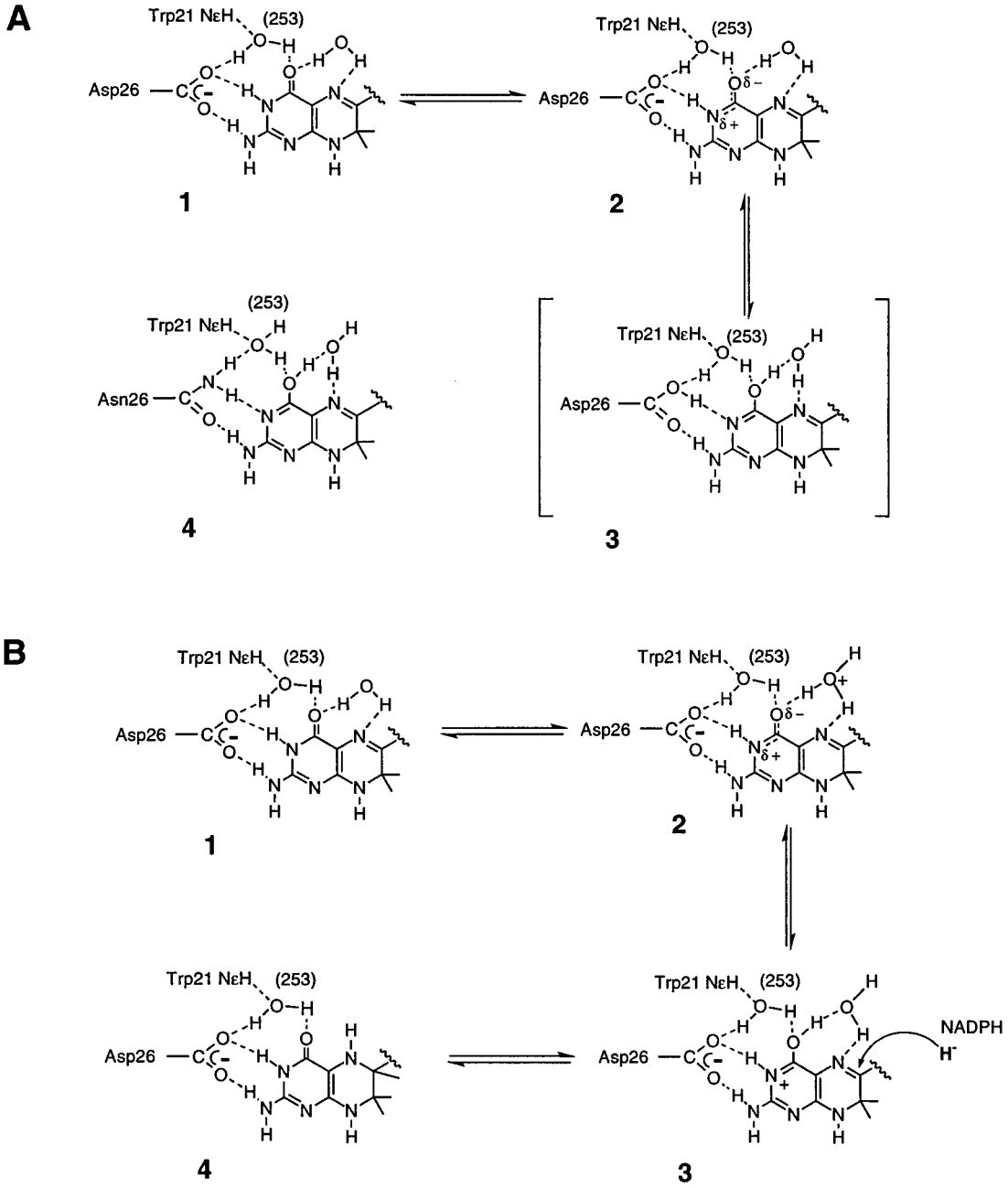

FIGURE 6: (A) Diagrammatic representation of structures of DHFR−FH_2 complexes: **1**−**3**, complexes with the wild-type enzyme, indicating the polarization of the substrate (**2**), which has been proposed by Cannon et al. (*23*) to lead to actual proton transfer to Asp26 and formation of the enol tautomer (**3**); **4**, complex with the Asp26Asn mutant with the enol tautomer of FH_2. (B) Postulated catalytic mechanism. Initial binding of FH_2 (**1**) is followed by polarization (**2**), as in A1-A2 above, and transfer of a proton from the solvent to O4 (**3**). Concerted proton transfer to N5 and hydride transfer to C6 lead to the keto tautomer of FH_4 (**4**).

The importance of the tautomeric form of the substrate in binding and catalysis by DHFR has received considerable attention in recent years (*8*, *9*, *23*, *27*−*29*). For example, the *L. casei* DHFR−folate−$NADP^+$ complex exists in three slowly interconverting states whose proportions are pH-dependent (*30*−*32*), and ^{13}C NMR studies of this complex using labeled folate have demonstrated the importance of substrate tautomerisation in determining the difference between these states (*27*). Recently, we have studied this complex with the [$^{13}C\gamma$-Asp]-labeled enzyme (*29*), allowing us to define the charge state of Asp 26 in the three states of the complex. We showed that at low pH, when folate was in the enol tautomeric state with N1 protonated, the most downfield $^{13}C\gamma$ peak in the spectrum of this complex arose from Asp 26, the low-field $^{13}C\gamma$ shift being attributed to the proximity of the positively charged N1−H. A low-field $^{13}C\gamma$ resonance was not evident for the keto tautomer of folate at higher pH values, where the Asp 26 side chain interacts with the N3H, which is not positively charged.

Direct comparison of the mode of binding of folate and FH_2 is inappropriate, since in the *L. casei* DHFR−folate−$NADP^+$ complex the enol form of folate binds with its pteridine ring turned over 180° relative to the "productive" orientation (*32*). However, it is noteworthy that a downfield $^{13}C\gamma$ resonance at a similar chemical shift position is observed for Asp 26 in the binary enzyme−FH_2 complex over the entire pH range. The presence of this downfield Asp 26-$^{13}C\gamma$ signal, shifted still further downfield in the

DHFR−FH_2−H_2NADPH complex, raises the possibility that the presence of the negative charge on Asp 26 polarizes the electron distribution of the bound FH_2 (in the keto tautomeric state; so as to create a partial positive charge at the N3 position, as shown in structure 2 in Figure 6A, leading to a deshielding of the Asp 26 carboxylate carbon atom). Some support for this polarization of bound FH_2 comes from the studies of Blakley et al. (*10*) on $^{13}C/^{15}N$-labeled folate and FH_2 binding to human DHFR. Both substrates bind in the keto tautomeric state, but the $^{15}N3$ resonance of folate shifts 8.06 ppm upfield on binding while the corresponding resonance of FH_2 shifts 4.25 ppm downfield on binding, clearly implying a different electron distribution in the two bound substrates. The proposed polarization of FH_2 such as to create a partial positive charge on N3, as shown in structure 2 in Figure 6A, could account for the observed deshielding of the nitrogen nucleus.

From crystal structure studies, the binding of NADPH analogues is known to induce closure of the "teen loop" (residues 9−23) (*33*, *34*), increasing the hydrophobic character of the binding pocket, and this appears to play an important role in catalysis. This would favor further neutralization of the negative charge of Asp 26 by further polarization of the N3−O4 system of FH_2. This is reflected in the spectrum of the DHFR−FH_2−H_2NADPH complex where a downfield resonance at 180.6 ppm is observed. In comparison to the binary FH2−enzyme complex, the rather large downfield shift for the Asp 26 resonance in this ternary complex (∼1.4 ppm) can be attributed to the combined effects of an increase in polarization and the enhanced hydrophobic environment established upon the addition of H_2NADPH. An increase in the polarization effect would lead to an increased partial negative charge at O4, which interacts both with the bridging water molecule (253) and with a second water molecule located between the teen loop, O4 and N5 atoms (*35*), as shown in Figure 6. We propose that this increased polarization of the substrate, driven by the hydrophobic nature of the binding site, would be sufficient to lead to protonation of O4 from the solvent, or at least to localization of a solvent proton near O4, leading to neutralization of the Asp 26-FH_2 system in the hydrophobic binding site, and that this proton transfer accounts for the apparent pK_a of 6.0 which describes the pH dependence of hydride transfer.

This suggestion is closely related to the catalytic mechanism recently proposed by Cannon et al. (*23*). They propose that the pK_a of 6.5 (*E. coli* DHFR) represents a transfer of the N3 proton from FH_2 to Asp 26, together with transfer of a proton from the solvent to O4, to form a neutral system consisting of a protonated (neutral) carboxyl group and FH_2 in the enol tautomeric state (structure 3 in Figure 6A). Our proposal differs from this only in that we suggest that the N3 proton is not transferred to the Asp 26 carboxylate; this receives clear experimental support from the $^{13}C\gamma$ chemical shift of the aspartate residue.

A proposed reaction mechanism for the reduction of FH_2 to FH_4, which is a modification of that proposed by Cannon et al. (*23*), is shown in Figure 6B. When the FH_2 molecule binds to the enzyme in the ternary complex, the polarization of the FH_2 molecule by Asp 26 favors transfer of a proton from solvent to O4, to create an N3-protonated enol species. A bound water molecule then provides a route for transfer of the proton from O4 to N5, concerted with hydride ion transfer to C6 from NADPH. The existence of this water molecule hydrogen bonding to O4 and N5 is supported by crystallographic studies (*35*); its lifetime and orientation is likely also to be controlled by the "teen loop".

This mechanism differs from that of Cannon et al. (*23*) in that it does not postulate transfer of a proton from FH_2 to Asp 26 to create the enol tautomer of the substrate. The NMR experiments reported here provide clear evidence that such a proton transfer does not occur in the enzyme−FH_2 binary complex, nor in the ternary complex if the enzyme−FH_2−H_2NADPH is a reasonable model of the catalytically functional ternary complex, although the possibility remains that this proton transfer does occur in the transition state. The effects of replacement of the active-site aspartate with an asparagine residue provide useful information in this context. In *E. coli* DHFR, the D27N mutation results in a decrease in k_{cat} to about 2% of the wild-type value at pH 6 (*5*), leading to the suggestion that a carboxylate group is essential for catalysis. However, the equivalent mutation in *L. casei* DHFR, D26N, results in a reduction in k_{cat} at pH 6 of only ∼6-fold (*36*), indicating that the presence of an aspartate at this position is by no means essential. In both cases, the pH dependence of k_{cat} (rate-limited by hydride transfer over the whole pH range) for the asparagine mutants is characterized by a modest and essentially linear decrease in activity with increasing pH (*5*, *36*), providing no evidence of the influence of an ionizable group. The simplest explanation for these observations is that the acetamido group of the asparagine side chain favors the enol tautomer throughout the entire pH range studied (structure 4 in Figure 6A), thus ensuring that O4 bears a proton across this pH range; the lower activity of the asparagine mutants might be explained by the different electron distribution in the species shown in Figure 6. The relatively high catalytic activity of *L. casei* DHFR D26N originally led us to favor a catalytic mechanism in which Asp 26 remains protonated throughout the catalytic cycle (*36*); however, the NMR observations reported here are clearly not consistent with this.

In conclusion, we believe that the available spectroscopic and kinetic data is consistent with the view that a major role of the active-site carboxylate in DHFRs is to polarize the bound substrate in such a way as to favor the enol tautomer. Experiments using FH_2 labeled with ^{13}C in a number of positions, together with mutagenesis studies, are currently underway to confirm the identity of the tautomeric species of dihydrofolate in its binary and ternary complexes with DHFR.

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